

Serum IgE Levels and *In Vitro* Production of IL-2, IL-4, and IFN- γ in Children with Down's Syndrome

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ABSTRACT

In nine children and one young adult with Down's syndrome (DS), lymphocyte phenotype, total serum IgE levels, and *in vitro* mononuclear cell (PBMC) proliferative responses to phytohemagglutinin (PHA) plus phorbol myristate acetate (PMA) and human recombinant interleukin-2 (hrIL2), and production of interleukin-2 (IL2), interleukin-4 (IL4), and interferon-gamma (IFN γ) were evaluated. The results showed a normal PBMC proliferative response to mitogens and a normal *in vitro* production of IL4 and IFN γ , while IL2 production was significantly decreased ($p < 0.05$). CD8+ cells were significantly increased ($p < 0.01$) and in five of eight patients with DS the IgE serum levels were lower than those in normal controls. IL4 production was lower in patients with DS with low IgE levels than in those with normal levels of serum IgE.

INTRODUCTION

VARIOUS IMMUNOLOGIC ABNORMALITIES have been reported in subjects with Down's syndrome (DS).⁽¹⁻⁴⁾ Some authors have shown low⁽⁵⁾ and others⁽⁶⁾ normal numbers of T-lymphocytes, a low CD4+ cell/CD8+ cell ratio,⁽⁷⁾ a weak⁽⁸⁾ or normal⁽⁹⁾ proliferative response to PHA. Antibody production also seems to be affected. IgG serum levels are often high, IgM and IgA levels are variable.^(10,11) It is possible that antibody deficiency in patients with DS could be due to the functional deficiency of T cells, which is attested to by anomalous thymic morphology⁽¹⁾ as well as low levels of thymic factors,⁽¹²⁾ and the various abnormalities mentioned above. The immune disorders could determine an increased susceptibility to bacterial and viral infections. On the contrary, it is not reported that the incidence of atopic diseases in subjects with DS is higher than in normals. The serum IgE levels have been found within normal limits⁽¹³⁾ or well below these.⁽¹⁴⁾ In this regard, it is known that *in vitro* IgE synthesis is regulated by the antagonistic effects of two cytokines, interleukin-4 (IL4) and interferon-gamma (IFN γ), the first stimulating and the second inhibiting IgE synthesis by mononuclear cells.⁽¹⁵⁾ The *in vivo* IgE synthesis and IL4 and IFN γ *in vitro* production are poor in the first years of life. The adult IFN γ *in vitro* production is reached at 3 years of age and over.⁽¹⁶⁾ The IL4 *in vitro* production has been reported, by Holt et al.,⁽¹⁷⁾ as similar to or significantly lower than that observed with adult T lymphocytes according to the culture methods used. The studies on IL4 and IFN γ production in subjects with DS are very few. Some authors have shown that the *in vitro* IFN production by DS lymphocytes after activation with PHA and polynosine-polycytidilic acid is decreased.⁽¹⁾

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Others have found higher levels of IFN γ mRNA in the thymus of children with DS than in those of controls.⁽¹⁸⁾ Finally, analysis of subjects with DS has revealed that induction of IFN γ mRNA was as vigorous as that observed for normal donors.⁽¹⁹⁾ By contrast, subjects with DS have shown a decrease in inducibility of interleukin-2 (IL2).⁽¹⁹⁾ To our knowledge, studies on IL4 production in patients with DS have not been published. It has been reported that IL4 induces a dose-dependent proliferative response of DS thymocytes like that observed with thymocytes of normal subjects.⁽²⁰⁾

The aim of our study was to evaluate the serum IgE levels in children with DS and its correlation with the *in vitro* production of IL4, IFN γ , and IL2. Peripheral blood mononuclear cell (PBMC) responses to phytohemagglutinin (PHA) plus phorbol myristate acetate ester (PMA) and to IL2, and phenotypic analyses of peripheral lymphocytes, were performed.

MATERIAL AND METHODS

Patients

Ten subjects (six male and 4 female; nine children with mean age $\pm$ SD 10.5 ± 5.2 years; range, 1–16 years; one 27-year-old) were enrolled in the study. Diagnosis of Down's syndrome was confirmed by karyotype analysis. Our patients were not institutionalized but were living with their families. They were examined as outpatients. The control group consisted of 11 healthy children (six boys and five girls; mean age $\pm$ SD, 10.1 ± 4.2 years; range, 1–14) to compare lymphocyte phenotype, serum IgE, and IL2 and IFN γ *in vitro* production and nine adults without atopic disease to compare IL4 *in vitro* production. Approval from the hospital's Ethics Committee was obtained.

Blood sampling and methods

Blood samples (10 ml) from subjects with DS were collected aseptically by puncture of the antecubital vein. An aliquot (1 ml) of every (but two) blood sample was centrifuged to obtain serum. The sera were stored at -20°C and then assayed by a radioimmunoassay kit (KABI Pharmacia Diagnostic AB, Uppsala, Sweden), unmodified, designed to measure IgE between 1.5 and 1000 kU/L on 0.1 ml of serum or plasma. The values obtained were compared with those normal for age.

Another aliquot (1 ml) of blood sample was placed into heparinized sterile tubes containing EDTA for lymphocyte phenotyping. Different monoclonal antibodies (MoAb) were used to characterize the phenotype of the cells. The MoAb and their CD nomenclature for human lymphoid differentiation antigens⁽²¹⁾ were as follows: OKT3 (CD3, panT cell, Ortho Diagnostic System), OKT4 (CD4, helper/inducer T cell, Ortho Diagnostic System), OKT8 (CD8, cytotoxic/suppressor T cell, Ortho Diagnostic System), B7 (CD19, panB cells, Beckton Dickinson). Flow cytometric analysis was performed using a fluorescent-activated cell sorter (Spectrum 3, Ortho Diagnostic System). The data were analyzed on an NEC 9801 VM, C51 program computer.

The last aliquot (8 ml) of the blood sample was placed into heparinized sterile tubes for leukocyte isolation. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque gradient centrifugation and then were washed three times with Ca^{++} and Mg^{++} -free phosphate-buffered saline (PBS). Thereafter, PBMC were resuspended at a concentration of 1×10^6 cells/ml in culture medium (RPMI 1640, Flow Laboratories, Irvine, Scotland), supplemented with antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin, Flow Laboratories, Irvine, Scotland), L-glutamine (2 mmol/L, Gibco Laboratories, Grand Island, NY) and containing 10% heated autologous human serum.

In 250 μl of culture medium 2.5×10^5 PBMC were placed into 96-well, flat-bottom tissue plates (Falcon 3070, Beckton Dickinson Labware, NJ). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3 days, in the presence of the following mitogens added to quadruplicate wells: PHA (100 $\mu\text{g/ml}$, M form, GIBCO Laboratories, Grand Island, NY) in combination with PMA (1 ng/ml, SIGMA, Milan, Italy); or human recombinant IL2 (hrIL2, 100 U/ml, Cetus Corporation, Emeryville, CA). Negative control wells received culture medium without mitogen. The supernatants of the cultures were harvested after 48 h of incubation and stored at -20°C until tested for lymphokine activity. The final volume of 250

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μ l was reconstituted with equal quantity of fresh culture medium, without mitogen. During the last 16 h of incubation, the cells were pulsed with 0.5 μ Ci/well of methyl[^{3} H]thymidine (6.7 Ci/mmol, NEN Products, Boston, MA). The cells were harvested onto glass fiber filter paper (Filtermats, Skatron Inc, Sterling, VA) using a multiple sample harvester (Cell harvester titertek, Flow Lab, Irvine, Scotland). The incorporation of thymidine into DNA was determined by liquid scintillation counter (Beckmann LS 7000) and radioactivity expressed as mean of quadruplicate cultures of counts per minutes (cpm) and stimulation index (SI: mean cpm of stimulated cultures/mean cpm of unstimulated cultures). Positive proliferative response was defined by SI >10.

In the PHA plus PMA-stimulated culture supernatants, IL2, IL4, and IFN γ were quantified by enzyme-linked immunosorbent assay (ELISA) kits (Intertest-2X, human interleukin-2 ELISA kit [Genzyme Corporation, Cambridge, MA], minimum sensitivity 100 pg IL2/ml; Intertest-4, human interleukin-4 ELISA kit [Genzyme Corporation, Cambridge, MA], minimum sensitivity 45 pg IL4/ml; Intertest-gamma, human interferon-gamma ELISA kit [Genzyme Corporation, Cambridge, MA], minimum sensitivity 100 pg IFN γ /ml). A 96 well microtiter plate, coated with monoclonal antibody specific for human IL2 (or IL4 or IFN γ), was used to capture IL2 (or IL4 or IFN γ) present in standard samples and unknown specimens. A goat polyclonal antibody that binds to multiple epitopes on the IL2 (or IL4 or IFN γ), contained on the solid phase was added. A third antibody, biotin-labeled donkey-antigoat Ig, which binds the goat polyclonal antibody already bound to IL2 (or IL4 or IFN γ), was then applied. Streptoavidin-peroxidase, which binds to the biotin on the third antibody, was added. Increased absorbance due to bound, immunoreactive IL2 (or IL4 or IFN γ) was quantitated by ELISA. The IL2 (or IL4 or IFN γ) concentrations in studied samples were determined by comparison of their absorbances with those obtained from the known amounts of human IL2 (or IL4 or IFN γ) in the standard dilutions.

Statistical analysis

Statistical analysis was carried out by Student's t-test and χ^2 test. Differences were considered significant when $p < 0.05$.

RESULTS

Table 1 summarizes the results of the tests performed on patients with DS.

Proliferative response to mitogens

PBMC of all patients with DS were stimulated with PHA + PMA. In nine of 10 we observed a positive response. One patient (BG) has shown an absent proliferative response to PHA + PMA (SI = 0.18) on two occasions. Excluding this subject, in patients with DS the mean $\pm$ SD of SI was 67.5 ± 48.8 and the range 10.5–147.3.

Proliferative response to hrIL2 has been evaluated in nine of 10 (90%) patients with DS (SI, mean $\pm$ SD 28 ± 41.9 ; range, 0.4–127.6). A positive response was observed in six of nine (66.6%) patients with DS.

IL2, IL4, and IFN γ in vitro production after PHA + PMA stimulation

IL2 was measured in five of 10 of patients with DS (Table 1) and in seven of 11 controls and was detectable in all of them. Patients with DS showed significantly ($p < 0.05$) lower mean values than controls (DS patients: mean $\pm$ SD 352 ± 92 pg/ml, range 320–520 pg/ml; controls: mean $\pm$ SD 935 ± 560 pg/ml, range 170–1850 pg/ml).

IL4 was detectable in six of 10 (60%) patients with DS and in eight of nine (88.8%) controls. No significant difference between the mean values was found (DS patients: mean $\pm$ SD 118.3 ± 71.9 pg/ml, range 45–245 pg/ml; control adults: mean $\pm$ SD 95 ± 36.1 pg/ml, range 54–175 pg/ml).

IFN γ was detectable in eight of 10 (80%) patients with DS and in all controls. Also for this cytokine the p calculated on the mean values of the two populations was not significant (DS patients: mean $\pm$ SD 805 ± 483.1 pg/ml, range 280–1700 pg/ml; controls: mean $\pm$ SD 747 ± 403.9 pg/ml, range 175–1350 pg/ml).

TABLE 1. STIMULATION INDEX (SI) TO PHA + PMA (P + P) OR IL2, SERUM IgE LEVELS AND IN VITRO PRODUCTION OF IL4, IFN γ , AND IL2 IN PATIENTS WITH DS

<i>Patients</i>	<i>Age (years)</i>	<i>SI P + P</i>	<i>SI IL2</i>	<i>IgE (ku/L)</i>	<i>IL4 (pg/ml)</i>	<i>IFNγ (pg/ml)</i>	<i>IL2 (pg/ml)</i>
CM	13	145.10	127, 56	3.1	130	350	320
GC	16	61.52	13, 43	3.7	75	400	370
CC	15	147.27	20, 22	n.d.	n.m.	1200	520
CA	14	9.87	1, 22	n.d.	45	280	250
DFR	3	55.19	11, 88	3.3	n.m.	n.m.	n.d.
ZE	1	43.79	62, 53	4.3	140	860	n.d.
BGL	10	73.02	3, 53	9.3	245	710	300
BG	11	0.18	0, 75	14.7	n.m.	n.m.	n.d.
PM	12	18.75	11, 32	6.4	75	940	n.d.
FG	27	52.66	n, d.	5.4	n.m.	1700	n.d.

n.d., not done; n.m., not measurable.

Determination of total serum IgE levels

In eight of 10 (80%) patients with DS we have determined the serum levels of total IgE (mean $\pm$ SD 6.2 $\pm$ 3.9 kU/L) and the obtained values were compared with those normal for age. Five of eight (62.5%) patients with DS showed low levels of serum IgE. In the 5 patients DS with low serum levels of IgE, IL4 was undetectable in two and, in the other three, mean $\pm$ SD of IL4 was 93.3 $\pm$ 31.8 pg/ml. In the three patients with DS with normal serum levels of IgE, IL4 was undetectable in one and, in the other two, mean $\pm$ SD of IL4 was 192.5 $\pm$ 74.2 pg/ml. IFN γ *in vitro* production was undetectable in both one patient with DS with low serum levels of IgE and in one patient with DS with normal serum level of IgE. When IFN γ was measurable, the mean $\pm$ SD was 847.5 $\pm$ 628 pg/ml in patients with low serum levels of IgE and 785 $\pm$ 106 pg/ml in patients with normal serum levels of IgE, without significant difference.

Phenotype analysis

No significant differences were found between the percentages of B cells, total T cells, and helper T cells in patients with DS (means $\pm$ SD: CD19+ cells, 8.5 $\pm$ 3.1; CD3+ cells, 75 $\pm$ 10.2; CD4+ cells, 37.2 $\pm$ 11.4) and in healthy children (CD19+ cells, 8.8 $\pm$ 2.1; CD3+ cells, 67.1 $\pm$ 8.5; CD4+ cells, 41.6 $\pm$ 5.1). The percentage of CD8+ T cells was significantly higher in patients with DS (40.7 $\pm$ 17.6) than in healthy subjects (23.2 $\pm$ 4.2) ($p < 0.01$).

DISCUSSION

It has been reported that in subjects with DS lymphocyte responses to PHA are normal during the first 10 years of age, but decline rapidly and irreversibly. This progressive impairment of the cellular immune responses is corroborated by evidence of alterations in immunologic function and defective lymphokine production *in vitro*.⁽²⁾ An explanation could be the low percentage of cells expressing high levels of CD3 compared with normal subjects.⁽²²⁾ The addition of PMA, which activates Ca⁺⁺/phospholipid-dependent enzyme protein-kinase C, or calcium ionophore A23187, which increases intracytosolic free Ca⁺⁺ concentration, enhanced, but did not normalize, the defective anti-CD2 mediated T-cell mitogenesis.⁽²³⁾ On the contrary, the proliferation of resting lymphocytes from trisomic patients was comparable to that of the control cells when PMA and A23187 were used as coblastogenic reagents.

Our patients with DS have shown on the whole normal responses to nonspecific blastogenic agents. In fact the percentage of subjects with reduced proliferative response did not significantly differ from what

we have found in the control adults. The percentage of CD3+ cells was within the normal range also for patient BG, who had showed poor proliferative responses to PHA + PMA and to hrIL-2. In conclusion, normal responses to mitogens in our subjects with DS could be due to normal expression of CD3 molecule on T cells and probably also to the young age of subjects examined. We point out that in the present study the PBMC were stimulated also with PMA, who, in association to A23187, normalize the defective anti-CD2-mediated T-cell from trisomic patients mitogenesis, as mentioned above.⁽²³⁾ It is possible that in the present investigation the association of PHA + PMA had a similar effect. Moreover, also other authors have found normal response to PHA in subjects with DS.⁽⁹⁾

In this study we have also explored the *in vitro* production of IL2, IL4, and IFN γ and the *in vivo* synthesis of IgE in children with DS. Gerez et al. have shown that analysis of subjects with DS revealed a pronounced decrease in the expression of the IL2 gene and IL2 mRNA in PHA-induced cultures with cycloheximide or after low-dose gamma irradiation.⁽¹⁹⁾ Previous studies have reported that subjects with DS show IFN mRNA production equal to or higher than that of control adults.⁽¹⁸⁾ Regarding the *in vitro* production of IL4, no study has been published today, even if it has been found that the proliferative response of DS thymocytes to IL4 is normal.⁽²⁰⁾

Our results show that IL2, *in vitro* production is significantly decreased in children with DS in agreement with the results of Gerez et al.⁽¹⁹⁾ and in contrast with other reports⁽²⁴⁾ that showed a normal IL2 synthesis. In our study the mean values of IL4 did not significantly differ between subjects with DS and controls. Our knowledge about this cytokine is poor in the healthy child. Holt et al. have observed, after stimulation with PHA + PMA, undetectable production of IL4 in T-cells' bulk cultures' supernatant. However, production of this cytokine was normal, with respect to control adults, in the supernatants of cultures obtained by cloning of T cells of studied children.⁽¹⁷⁾ The authors conclude that culture conditions employed during cloning can radically influence the cytokine production capacity of CD4+ T-cells. IL4 production is stimulated by the cloning process per se, since all bulk culture supernatants tested fell at the extreme low end of the range observed with adults.

It is possible that our children with DS have reached functional maturity regarding IL4 *in vitro* production, in agreement with the observed normal response to mitogens. On the other hand, the age of our patients probably was not so advanced to justify a profound immunodeficit.

In supernatants of cultures from our subjects with DS the levels of IFN γ were comparable to those of controls. This is in keeping with the normal or higher IFN γ mRNA production observed in the thymus of patients with DS than in control subjects.⁽¹⁸⁾ On the contrary, our results are in seeming contrast with other studies that reported a low IFN production by DS lymphocytes after PHA and polynosine-polycytidilic acid stimulation.⁽¹⁾ However, it is difficult to compare the studies on cytokine production since the various authors have used different methods of stimulation and culturing.

The serum IgE levels in patients were lower than those in normals. Low serum IgE levels have been reported in some studies,⁽¹⁴⁾ and normal values in others.⁽¹³⁾ It was interesting to verify the existence of a relation between *in vivo* serum IgE levels and *in vitro* production of IL4 and IFN γ . It seems that patients with DS with low IgE serum levels *in vitro* produce lower levels of IL4 than patients with DS with normal serum levels of IgE, even if the difference is not significant. Lack of significance could be due to the small size of the study population. On the other hand, T cells of atopic subjects with high IgE serum levels produce levels of IL4 and IFN γ comparable to those produced by T cells of normal controls after *in vitro* PMA plus CaCl₂ stimulation. Only when a specific allergen is applied as stimulus to T cells of atopic subjects produce higher levels of IL4 and lower levels of IFN γ than normals.⁽²⁵⁾

Levels of CD8+ cells were higher in patients with DS than in controls ($p < 0.01$). Their role in IgE synthesis is unknown and, generally, the mechanisms of suppression are undefined. Culture supernatants of CD8+ cell clones contain soluble factors, so far uncharacterized, with suppressing activity. Moreover, some of the activities of suppressor T cells may be explained by indirect mechanisms, for example, the IL2 consumption.⁽²⁶⁾ In our patients, it is possible to hypothesize a connection between a high percentage of CD8+ cells and low *in vitro* detectable levels of IL2. IL2 is a pleiotropic cytokine with a fundamental role in B-cell proliferation and differentiation and in antibody synthesis.⁽²⁷⁾ However, we can not affirm with certainty that these features are responsible for the low levels of serum IgE in children with DS.

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